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Essential function of nitric oxide synthase in *Drosophila*

Michael Regulski*, Yuri Stasiv, Tim Tully and Grigori Enikolopov*

Nitric oxide (NO), produced by NO synthases (NOS), is a short-lived intra- and transcellular messenger that regulates many physiological functions in vertebrates and invertebrates (e.g., blood pressure, muscle contraction, innate immunity, cell division and differentiation, response to hypoxia, and memory formation [1]). Given its numerous functions, it might be expected that a lack of NOS activity would be lethal for the developing organism. However, such an indispensable role for NO in development has not yet been demonstrated. A genetic analysis of NO function in vertebrates is complicated by the presence of three NOS genes. Mice with a homozygous ablation of any single NOS gene are viable, animals with two NOS genes knocked out show drastically reduced viability and triple knockout animals have not yet been generated [2–6].

To test whether NOS function is essential for the developing organism, we performed a genetic study in *Drosophila,* which carries a single NOS gene (*dNOS*) [7,8]. The *dNOS* locus encodes a family of proteins including DNOS1, a full length enzymatically active form, which bears extensive similarity to its mammalian sequelogs [9], and whose activity is dependent on the same cofactors as vertebrate NOS proteins. *dNOS1* is first expressed in the embryo and later in the larva, pupa and adult.

Our experimental approach was to generate point mutations and test them against a deficiency in the *dNOS* locus. To generate the deficiency, we mobilized the *l(2)k08405* P-element located 11 kb proximal to *dNOS*. This produced a 20 kb deletion, *Df(2L)69F*, which is homozygous lethal and spans part of the *dNOS* transcription unit along with five flanking genes (Figure 1). Flies heterozygous for this deletion showed reduced levels of DNOS protein and half the wild-type level of NOS activity (Figure 1B,C). No novel NOS-related peptides or transcripts were revealed by Western and Northern analyses of flies bearing the deficiency chromosome (data not shown). We next generated 4855 mutant lines, using ethyl methanesulfonate (EMS), and screened them for lethality by complementation with the deficiency chromosome. 32 lethal mutations, falling into five complementation groups, were identified (Supplemental Data). Extracts from one of these mutants, $dNOS^{C}$, revealed reduced levels of NOS enzymatic activity (Figure 2), suggesting that this strain carries a null mutation in *dNOS*. We performed five generations of outcrossing of the mutant to wild-type flies, replacing at least 95% of the original mutated genome [10], and found that in this new genetic background the mutated gene still conferred larval lethality to homozygotes, indicating that the effect is not due to a secondary mutation. Sequence analysis of genomic DNA from the $dNOS^{C}$ strain identified a single G to A transition corresponding to position 1942 of the *dNOS1* mRNA, creating a glycine (Gly) to glutamic acid (Glu) substitution at position 585 of DNOS1 (Figure 2B). We expressed the mutated DNOS1 protein in cell culture and found that its enzymatic activity was abolished even though the protein was expressed at the same level as wild-type DNOS1 in this assay (Figure 2C,D). Flies homozygous for the DNOSGly585Glu mutation die during late embryonic and larval stages, demonstrating that DNOS

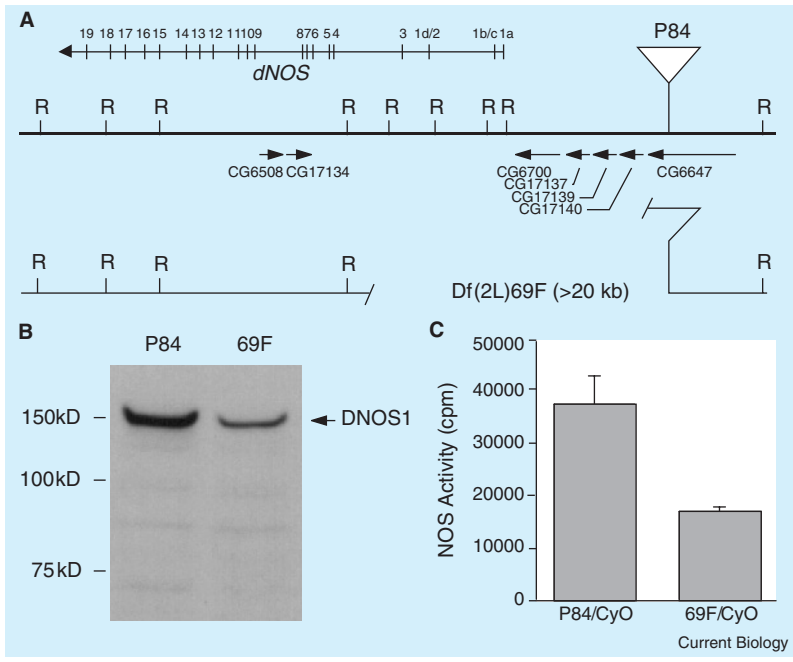

Figure 1. The *dNOS* gene is disrupted in the *Df(2L)69F* chromosome.
(A) Genes within 50 kb of the *Drosophila* genome surrounding *dNOS* and mitochondrial porin genes. Arrows depict transcription units. The *dNOS* transcription unit is indicated by the top arrow and vertical bars indicate approximate location of *dNOS* exons. CG6508 and CG17134 are cathepsinD-like genes nested within intron 8 of *dNOS*. CG17137, CG17139 and CG17140 refer to three genes with strong homology to mitochondrial porin, CG6647. The triangle shows the location of the *l(2)k8405* P-element insertion, which was used to generate the deficiency *Df(2L)69F* shown in the bottom part of the figure. 'R' = *Eco*RI sites. (B) Western blot: head protein extracts (50μg/lane) prepared from *Df(2L)69F/CyO (69F)* show a decreased level of DNOS1 protein compared to the parental line *l(2)k8405/CyO (P84)*. The Western blot was probed with mAb 6/157 raised against the carboxyl-terminus of DNOS. (C) NOS activity in head extracts (20 μg/lane) prepared from deficiency *Df(2L)69F/CyO* or parental *l(2)k8405*/CyO heterozygotes.

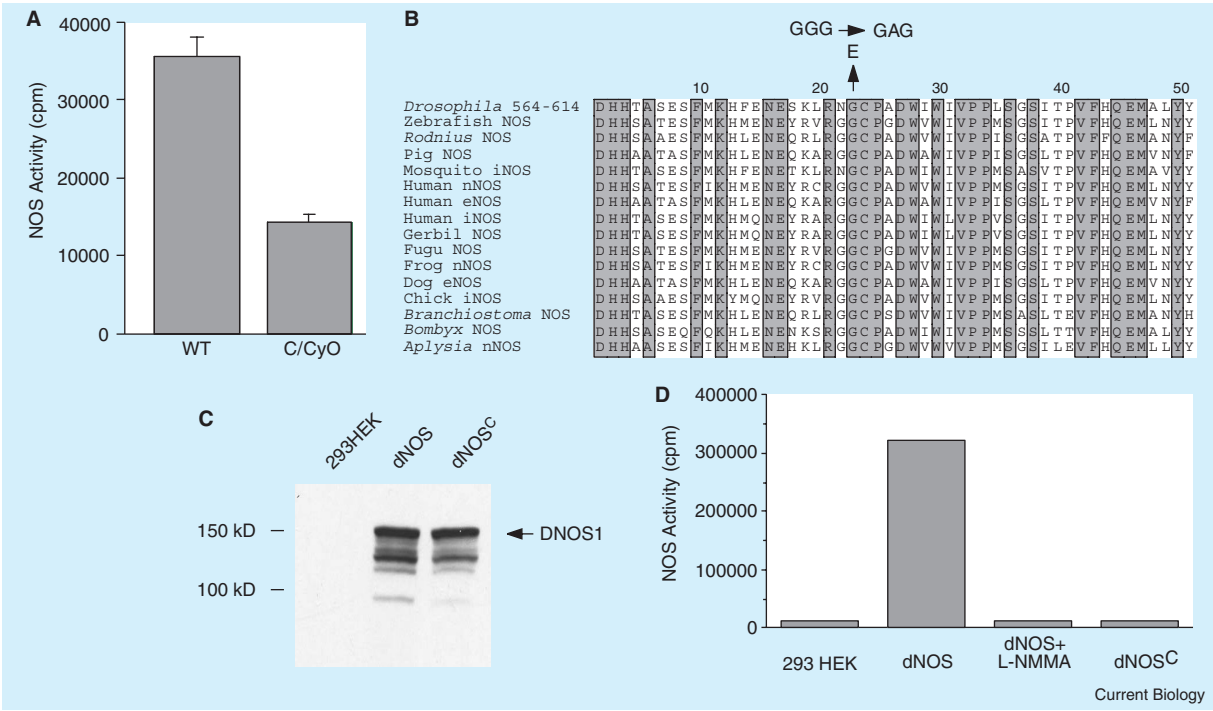

Figure 2. *dNOS^{C}* is a null allele of the *dNOS* gene.
(A) NOS activity measured in head extracts prepared from wild-type flies (WT; *w^{1118} (CJiso1))* or *dNOSC/CyO (C/CyO)* is decreased in the heterozygote. (B) Alignment of the region surrounding the *dNOS^{C}* mutation with corresponding regions from a selected group of NOS proteins shows that *dNOS^{C}* affects a conserved glycine. (C) Western blot analysis of cell culture extracts (293 human embryonic kidney cells; 293 HEK) indicates that wild-type (dNOS) and mutant (dNOS^{C}) DNOS proteins are expressed at comparable levels. (D) Enzymatic assay: NOS activity, measured in the same extracts as in (C), is absent from the mutant protein as compared with the control. dNOS + L-NMMA, which contains 1 mM NOS inhibitor L-NMMA, indicates the baseline activity in this assay.

is required for normal *Drosophila* development. The mutated glycine residue is conserved across all characterized NOS proteins (Figure 2B) and appears to be located at an invariant position in the three-dimensional structure (based on the crystal structure of mammalian NOS). Although Gly585 does not map to any previously identified functional domain of NOS, it appears to play a structural role. This residue cannot be replaced without significant perturbation of the local protein structure given that the glycine ϕ,ψ-angles fall in disallowed regions of the Ramachandran plot.

The 16 other lethal mutations in the *dNOS* complementation group that we have generated will help to define other critical functional domains of NOS. Our results in *Drosophila* provide the first evidence that NOS function is essential for development. In mammals, the effect of a NOS deficiency may be masked by the compensatory action of other NOS isoforms, obscuring the true importance of NO signaling in normal development and physiology.

Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/14/20/R881/DC1/

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Cold Spring Harbor Laboratory, 1 Bungtown Rd., P.O. Box 100, Cold Spring Harbor, NY 11743, USA. E-mail: regulski@cshl.edu; enik@cshl.edu